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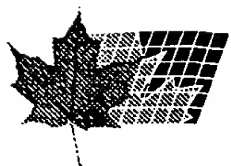
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(54) **CYCLODEXTRINE-GLYCOSYL-TRANSFERASES POUR
PRODUIRE DE LA GAMMA-CYCLODEXTRINE**
(54) **CYCLODEXTRIN GLYCOSYL TRANSFERASES FOR
PRODUCING .GAMMA-CYCLODEXTRIN**

(57) CGTases, qui, lorsqu'elles convertissent des substrats amidonnés ou semblables à l'amidon en cyclodextrines, produisent de la gamma-CD dans une proportion plus élevée et continuent de présenter au moins 60 % de l'activité CGTasique spécifique totale de la CGTase de départ utilisée pour préparer l'enzyme en question. Les séquences d'acides aminés diffèrent de celles des CGTases connues par la délétion d'au moins un acide aminé dans la région allant de la position 155 jusqu'à la position 195 inclusivement, la position 1 de la séquence de la protéine étant le début du peptide signal de la CGTase, et la délétion entraîne une augmentation de l'activité gamma-CGTasique de la protéine.

(57) CGTases, which, when converting starch or starch-like substrates to CD, produce .gamma.-CD to an increased extent and still exhibit at least 60% of the specific total CGTase activity of the starting CGTase which was used for preparing the enzyme concerned. The amino acid sequences differ from the amino acid sequences of known CGTases by the deletion of at least one amino acid from the region from amino acid position 155 up to and including amino acid position 195, where position 1 of the protein sequence is the beginning of the signal peptide of the CGTase and the deletion brings about the increase in the .gamma.-CGTase activity of the protein.

CYCLODEXTRIN GLYCOSYL TRANSFERASES FOR PRODUCING γ -CYCLODEXTRIN

ABSTRACT OF THE DISCLOSURE

CGTases, which, when converting starch or starch-like substrates to CD, produce γ -CD to an increased extent and still exhibit at least 60% of the specific total CGTase activity of the starting CGTase which was used for preparing the enzyme concerned. The amino acid sequences differ from the amino acid sequences of known CGTases by the deletion of at least one amino acid from the region from amino acid position 155 up to and including amino acid position 195, where position 1 of the protein sequence is the beginning of the signal peptide of the CGTase and the deletion brings about the increase in the γ -CGTase activity of the protein.

2207—c.spc

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to cyclodextrin glycosyl transferases (CGTases) EC 2.4.1.19 for producing γ -cyclodextrin, to processes for preparing γ -cyclodextrin glycosyl transferases, and to their use.

2. The Prior Art

As a rule, cyclodextrins are prepared from starch or starch-like substrates. In these preparations, CGTases are used to convert starch enzymically into cyclodextrin (CD). For thermodynamic reasons, the starch is mainly converted into β -CD, independently of the CGTase used for the reaction, if the reaction is carried out until the thermodynamic equilibrium is reached (maximum CD yield). However, in the initial phase, at the beginning of the starch conversion reaction, the enzymes which are used for the conversion differ in the composition of the primary product mixture. α - β - or γ -CGTases are differentiated depending upon the product, α -, β -, or γ -CD, which is chiefly formed by the enzyme in this initial phase.

These enzymes, which are suitable, and have also already been used, for the industrial production of CD, have hitherto only been detected in bacteria. α -CGTases have hitherto only been identified in *Bacillus macerans*, *Bacillus stearothermophilus* and *Klebsiella oxytoca*. β -CGTases have been detected, for example, in *Bacillus circulans*, *Bacillus megaterium*, *Bacillus ohbensis*, *Micrococcus sp.* and alkalophilic *Bacillae* which have not been precisely classified taxonomically, such as *Bacillus sp.* 38-2, 17-1, 1011 or 1-1. Naturally occurring enzymes having an initially high γ -CD-forming activity have been reported in *Bacillus subtilis* 313, *Bacillus sp.* A1-6 and *Bacillus sp.* 290-3.

Since the CGTases which are used in the industrial preparation of cyclodextrins always yield mixtures of several cyclodextrins when converting starch into cyclodextrins, various processes have been developed for isolating pure cyclodextrins (α , β or γ). These are described below:

Defined CDs can be separated out chromatographically from the product mixtures, e.g. on the basis of differences in their molecular weights (described, for example, in US Patent No. 4,808,232).

As a rule, when starch is converted enzymically into cyclodextrins, complexing agents are added which only react with one defined CD and with this CD form an insoluble complex, for example, which can then be separated out from the reaction mixture by physical means. Subsequently, the complex is resolved and the homogeneous CD is isolated (described, for example, in EP 0291067).

When a γ -CGTase is used, the product composition can be displaced in the γ -CD direction by adding an organic solvent, such as ethanol, to the reaction mixture (*J. Ferm. Bioeng.* (1990) 70 (3), pp. 150-154).

In each of the processes, those CGTases are optimally used which possess an initial product formation preference which is as high as possible for the CD which is to be prepared in pure form.

The specificity of the previously known α - and β -CGTases is adequate for industrial production of the corresponding cyclodextrins. By contrast, none of the known, naturally occurring γ -CGTases possesses a product specificity which permits a comparable industrial production of γ -CD.

In order to prepare γ -CD, therefore, it was proposed, in CA 115:157165, that α - and/or β -cyclodextrins be converted enzymically into γ -CD by adding the γ -CD-specific complexing agent glycosyl glycyrrhizin, maltose and a CGTase.

Another option for preparing γ -CD consists in increasing the γ -CD specificity of β -CGTase, by means of exchanging defined amino acid residues, to such a degree that the mutagenized enzyme produces γ -CD to an increased extent and can consequently be used for preparing γ -CD on an industrial scale. Appropriate mutations are known and described, for example, in DE 43 24 650 A1 (corresponds to US Patent No. 5,474,917), *Biochemistry* (1994) 33 (33), pp. 9929-9936, *Biochemistry* (1995) 34 (10), pp. 3368-3376 and *J. Biotech.* (1994) 32, pp. 283-288.

Such CGTase derivatives, which have been produced by mutagenizing β -CGTases, possess an increased specificity for γ -CD and are consequently, on the basis of their product spectra, suited, in principle, for the industrial preparation of γ -CD. However, a disadvantage is that the specific activities of the starting enzymes which are used for the mutagenesis are reduced by introducing the relevant mutations. In dependence on the amino acid residues which are introduced, mutated enzymes having an

increased specificity for γ -CD only possess between 25% and 50% of the CD-forming activity of the starting enzyme (*Biochemistry* (1994) 33 (33), pp. 9929-9936, *Biochemistry* (1995) 34 (10), pp. 3368-3376).

SUMMARY OF THE INVENTION

It is an object of the present invention to provide cyclodextrin glycosyl transferases (CGTases) which, when converting starch or starch-like substrates into CD, produce γ -CD to an increased extent and which still exhibit at least 60% of the specific total CGTase activity of the starting CGTase which was used for preparing the enzyme concerned.

An additional object of the present invention is to provide processes for preparing the said CGTases.

A further object of the present invention is to provide a process for producing γ -CD.

The first-mentioned object is achieved by CGTases whose amino acid sequence differs from the amino acid sequence of wild-type CGTases by the deletion of at least one amino acid in

the region from amino acid position 155 up to and including amino acid position 195, where position 1 of the protein sequence is the beginning of the signal peptide of the CGTase and the deletion increases the γ -CGTase activity of the protein.

Within the meaning of the invention, increases in the γ -CGTase activity is understood to mean that the quotient

$$\frac{\text{quantity of } \gamma\text{-CD formed}}{(\text{quantity of } \alpha\text{-CD formed} + \text{quantity of } \beta\text{-CD formed})}$$

becomes greater in the product mixture which arises when starch or starch-like substrates are reacted with CGTases.

Preferably, the amino acid sequences of CGTases according to the invention differ from the amino acid sequences of known CGTases by between four and eight amino acid residues being deleted in the region between amino acid position 155 and amino acid position 195 of their protein sequence, where position 1 of the protein sequence is the beginning of the signal peptide of the CGTase and the deletion increases the γ -CGTase activity of the protein.

Particularly preferably, the amino acid sequences of CGTases according to the invention differ from the amino acid sequences of known CGTases by six amino acid residues being deleted in the region between amino acid position 155 and amino acid position 195 of their protein sequence, where position 1 of the protein sequence is the beginning of the signal peptide of the CGTase and the deletion increases the γ -CGTase activity of the protein.

It also applies for each of the other amino acid positions mentioned in the application that position 1 of the protein sequence is the beginning of the signal peptide of the CGTase.

In addition, CGTases are in particular preferred whose amino acid sequences differ from the amino acid sequences of the CGTases specified in Table 1 and FIG. 1 at least by the deletion of the amino acid residues which are in each case printed in bold, with the remaining amino acid sequence of the respective CGTase according to the invention being homologous to the amino acid sequence of the CGTase specified in Table 1 and FIG. 1 to the extent that the sequence exhibits CGTase activity without the deletion according to the invention.

Examples of CGTases according to the invention are CGTases which are obtained from the CGTases listed in Table 1 and FIG. 1, or from other CGTases, by deleting individual amino acid residues in the region between the amino acid residues 155 and 195. CGTases are preferred in which from four to eight residues have been deleted from the said region. CGTases are particularly preferred in which the six amino acids marked by bold type in Table 1 and FIG. 1 have been deleted from the said region.

Further examples of CGTases according to the invention are enzymes from which the amino acids which are homologous to the amino acids specified in Table 1 and FIG. 1 have been deleted, with these enzymes exhibiting CGTase activity without the deletion according to the invention.

Further, examples of CGTases according to the invention are enzymes in which the amino acid residues which are in each case printed in bold in FIG. 1 have been deleted from the region between amino acid position 155 and amino acid position 195, with the remaining amino acid sequence of the CGTases according to the invention being homologous to the amino acid sequence of the CGTase from the microorganism which is in each case specified in FIG. 1 and Table 1 to the extent that the

enzyme whose sequence does not contain the deletion according to the invention exhibits CGTase activity.

The six amino acids residue of (SEQ ID NO:5) is that deleted portion found in (SEQ ID NO:4). The six amino acids residue of (SEQ ID NO:7) is that deleted portion found in (SEQ ID NO:3). The six amino acids residue of (SEQ ID NO:9) is that deleted portion found in (SEQ IS NO:2). The six amino acids residue of (SEQ ID NO:10) is that delete dportion found in (SEQ ID NO:1).

The CGTase protein of the invention has an amino acid sequence which differs from the amino acid sequences of the CGTases specified in FIG. 1 and Table 1 at least by the deletion of the amino acid residues which are in each case printed in bold in FIG. 1, and Table 1 and is selected from the group consisting of:

(SEQ ID NO:5) deleted from (SEQ ID NO:4);
(SEQ ID NO:7) deleted from (SEQ ID NO:3);
(SEQ ID NO:9) deleted from (SEQ ID NO:2); and
(SEQ ID NO:10) deleted from (SEQ ID NO:1).

The remaining amino acid sequence of the CGTase according to the invention is homologous to the amino acid sequence of the CGTase from the microorganism which is in each case specified in FIG. 1 to the extent that the sequence exhibits CGTase activity without the deletion according to the invention.

TABLE 1:

<u>β-CGTase from</u>	<u>Position</u>	<u>Amino acid sequence</u>	<u>Six Deleted Amino Acid Residue</u>
<i>Bacillus ohbensis</i>	160	(SEQ ID NO:1)	(SEQ ID NO:10)
<i>Bacillus macerans</i>	165	(SEQ ID NO:2)	(SEQ ID NO:9)
<i>Bacillus</i> sp. 1-1	160	(SEQ ID NO:3)	(SEQ ID NO:7)
<i>Bacillus circulans</i> #8	172	(SEQ ID NO:4)	(SEQ ID NO:5)

Unexpectedly, the CGTases according to the invention possess a higher γ -CD specificity than that of the starting CGTases which were used for their preparation while, at the same time, the mutated enzyme only exhibits an insignificant reduction in specific total CGTase activity as compared with that of the starting CGTase.

When converting starch or starch-like substrates, the CGTases according to the invention consequently produce CDs in a product distribution in which the quotient of γ -CD and the sum of α -CD and β -CD is greater than the quotient of these products which is obtained when starch is converted using the respective unaltered starting CGTase.

The list shown in Table 1 and in FIG. 1 shows, using a few CGTases by way of example, the homologous amino acid sequence region which is generally present in CGTases and the six amino acid residues within this sequence region which are in each case relevant for modifying the product specificity.

The four amino acid sequences shown in FIG. 1 are the same respectively as the four amino acid sequences shown in Table 1.

The number of the first amino acid of each of the amino acid sequences depicted in Table 1 and in FIG. 1 is designated as the position, with the first amino acid of the signal peptide of the particular CGTase sequence having been counted as position 1. The corresponding sequence region can be found in all CGTases using well known standard methods. This can be done, for example, using known algorithms which calculate multiple sequence alignments. An example of a suitable computer algorithm is the "pileup" program from the commercially available Wisconsin Sequence Analysis package (Genetic Computer Group, Madison, Wisconsin) sequence analysis program.

By means of mutagenizing the depicted region in CGTases, enzymes according to the invention can be prepared from any CGTases using known standard methods, as explained, by way of

example, in the present application. For this purpose, a gene encoding a CGTase is as a rule mutated in such a way that it then encodes a CGTase according to the invention.

The invention consequently also relates to processes for preparing mutated CGTase genes which encode CGTases according to the invention, wherein the DNA sequence of a gene encoding a starting CGTase is mutated, by means of mutagenesis methods which are known per se, such that the amino acid sequence in the region between amino acid positions 155 and 195, which is encoded by the DNA sequence of the mutated gene, differs from the amino acid sequence which is encoded by the DNA of the unmutated gene by the deletion of at least one amino acid residue.

Preferably, in the process according to the invention, the DNA sequence of a gene encoding a starting CGTase is mutated, by means of mutagenesis methods which are known per se, such that the amino acid sequence encoded by the DNA sequence of the mutated gene differs from the amino acid sequence encoded

by the DNA of the unmutated gene by the deletion of from four to eight amino acid residues from the region between amino acid positions 155 and 195.

Particularly preferably, in the process according to the invention, the DNA sequence of a gene encoding a starting CGTase is mutated, by means of mutagenesis methods which are known per se, such that the amino acid sequence encoded by the DNA sequence of the mutated gene differs from the amino acid sequence encoded by the DNA of the unmutated gene by the deletion of six amino acid residues from the region between amino acid positions 155 and 195.

The invention furthermore relates to processes for preparing γ -CGTases, wherein at least one of the described DNA sequences is expressed in a microorganism.

The genes of all CGTases (starting CGTases) are suitable for preparing the CGTases according to the invention.

While starting CGTases can be all naturally occurring CGTases, they can also be CGTases which are obtained by mutagenesis, for example those CGTases in which the product formation ratio has already been altered by another mutation which is not in accordance with the invention (e.g.: as in DE 43 24 650 A1, which corresponds to US Patent No. 5,474,917). Starting CGTases are preferably those CGTases in which the product formation ratio has already been altered by another mutation which is not in accordance with the invention (e.g.: as described in DE 43 24 650 A1).

The gene encoding a starting CGTase is isolated using known methods and the mutation according to the invention is introduced into the gene of the CGTase by "in-vivo" or "in-vitro" mutagenesis methods. These methods are likewise well known in the state of the art.

"In-vivo" mutagenesis methods are to be understood as being, in particular, those methods in which microorganisms

which chromosomally and/or episomally contain a gene encoding a CGTase are mutagenized in a non-specific manner with a mutagen such as UV light, nitrosoguanidine or ethyl methyl sulfonate. Such a method is described, for example, by Miller J. H. in (1972) *Experiments in Molecular Genetics*; Cold Spring Harbor Laboratory; Cold Spring Harbor, N.Y..

Subsequently, known methods, such as sequence analysis in accordance with the chain termination method described by Sanger et al. in PNAS 74 (1977) 5463-5467 are used to identify mutants in which at least one codon of the CGTase gene, encoding an amino acid residue, has been deleted from the region between amino acid residues 155 and 195 of the corresponding CGTase.

Those mutants are preferably selected in accordance with the invention in which from four to eight codons have been deleted from the said region.

Mutants are particularly preferably selected in which six codons have been deleted, with those mutants once again preferably being selected in which the six codons have been deleted which encode the amino acid residues which are printed in bold in FIG. 1 or encode the amino acid residues which are homologous to these residues in other CGTases.

Within the meaning of the invention, "in-vitro" mutagenesis methods are to be understood as being those methods in which an isolated CGTase gene, or a fragment of a CGTase gene, is modified, in a manner known per se, such that a gene is produced which encodes a CGTase enzyme in which at least one codon of the CGTase gene, encoding one of the amino acid residues in the region between amino acid residues 155 and 195, has been deleted.

Mutants are preferred which have been modified such that from four to eight codons have been deleted in the said region. Mutants are particularly preferred which have been

modified such that six codons have been deleted in the said region. In particular, mutants are particularly preferred which have been modified such that, in the said region, the six codons have been deleted which encode the amino acid residues which are printed in bold in FIG. 1 or encode the amino acid residues which are homologous to these residues in other CGTases.

The invention consequently also relates to DNA sequences which encode γ -CGTases according to the invention.

Examples of methods for "in-vitro" mutagenesis which are known from the state of the art are specific (*BioTechniques* (1992) 13 (3), pp. 342-346) or non-specific (*Technique* (1989) 1 (1), pp. 11-15) mutagenesis methods which use the "PCR" technique. Methods are also known in which the mutation is introduced into the target gene in a directed manner using a synthetic oligonucleotide. This can take place either using so-called "single-strand methods" (Ausubel F.M. et al. (1987) *Current Protocols in Molecular Biology*, Green Publishing

Associates) or using "double-strand methods" (*Promega 1992-1993 Catalogue*, 150) or using other methods as described, for example, in *Ann. Rev. Genet.* (1985) 19, pp. 423-462.

The main area of application for the CGTase according to the invention is its use for isolating γ -CD from starch. The CGTases according to the invention can be employed for this purpose using current preparation methods.

The invention consequently also relates to processes for preparing γ -CD by converting starch using a CGTase, wherein at least one CGTase according to the invention is employed as the CGTase.

Current preparation methods for producing γ -CD, in which the CGTases according to the invention can be employed in place of the CGTases which are specified in these methods, are described, for example, in:

- *Journal of Fermentation and Bioengineering*
(1990) 70 (3), pp. 190-192: The preparation of γ -CD using the β -
and γ -CD-forming CGTase from *Bacillus sp. AL-6* in the presence of
ethanol, which results in an increased production of γ -CD.

- CA 107:57466 describes the preparation of γ -CD
using the γ -CGTase from *Bacillus sp. 313*.

- EP 291,067: Preparation of γ -CD using the
CGTase from *Bacillus macerans*. Product specificity for γ -CD is
achieved by adding a complexing agent, for example cyclohexadec-
8-en-1-one.

- DE 40 09 822 (corresponds to US Patent No.
5,409,824): Production of γ -CD using the γ -CGTase from *Bacillus*
sp. 290-3.

Both in comparison to α -CD and in comparison to β -CD, γ -CD possesses specific advantages which identify it as the only possible CD, or the most suitable CD, for a series of applications.

In comparison to α -CD, which is made of six glucose units, γ -CD, which consists of eight glucose units, possesses a larger hydrophobic cavity which also makes it possible to complex guest molecules which, for steric reasons, cannot be complexed by α -CD.

In comparison to β -CD (solubility in water at room temperature: approx. 18.5 g/l), γ -CD possesses a substantially higher solubility in water (at room temperature: approx. 232.0 g/l) and is consequently more suited than β -CD for complexing reactions in aqueous solutions. A further advantage of γ -CD, when compared with β -CD and modified β -CD derivatives, is its low toxicity. In an animal model, α -CD derivatives and β -CD

derivatives are more toxic than γ -CD when administered either orally or intravenously.

Other objects and features of the present invention will become apparent from the drawing and from the following Examples, which disclose the embodiments of the present invention. It should be understood, however, that the drawing and the Examples are designed for the purpose of illustration only and not as a definition of the limits of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawing, FIG. 1 shows the homologous amino acid sequence region which is generally present in CGTases and the six amino acid residues in bold print within this sequence region which are in each case relevant for modifying the product specificity.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

EXAMPLE 1

Mutagenesis of the *Bacillus circulans* #8 (DSM 10559) CGTase

Deletion of any amino acid residues in the region, according to the invention, of amino acid residues 155-195, in particular deletion of the six amino acid residues (SEQ ID NO:5) at positions 179-184 in the β -CGTase from *Bacillus circulans* #8 (see Table 1; deposited March 2, 1996, in the DSMZ Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures) in Mascheroder Weg 1b, D-38124 Braunschweig, Germany, under number DSM 10559 according to the Budapest Treaty), is achieved by deleting, in a manner which is known per se to a person skilled in the art, the base triplets of the CGTase structural gene which encode the corresponding amino acid residues.

For the mutagenesis, the β -CGTase gene from *Bacillus circulans* #8 was first of all cloned into the commercially available *E. coli* vector pUC19 (Boehringer, Mannheim). For this purpose, chromosomal DNA was isolated from *Bacillus circulans* #8 (Appl. Microbiol. Biotechnol. (1990) 33: pp. 542-546) as described in Ausubel F.M., *Current Protocols In Molecular Biology*, Vol. 1; Greene Publishing Associates & Wiley - Interscience, N.Y. and cleaved with the restriction endonucleases HindIII and XbaI (Boehringer, Mannheim). Fragments in a size range of between two and five kb were isolated and incubated, at 16°C for 12 hours, together with pUC19 DNA, which had been cleaved with the restriction endonucleases HindIII and XbaI (Boehringer, Mannheim), and with T4 DNA ligase. The ligation mixture was used to transform *E. coli* K 12 cells which had been rendered competent for DNA uptake by means of known methods (Maniatis, *Molecular Cloning, A Laboratory Manual*; Cold Spring Harbor Laboratory (1982), N.Y.). The recombinant plasmid, which carries the gene for the *Bacillus circulans* #8 β -CGTase, was isolated from those *E. coli* cells which, following the

transformation, formed starch degradation haloes on starch-containing indicator plates (Maniatis, *Molecular Cloning, A Laboratory Manual*; Cold Spring Harbor Laboratory (1982), N.Y., pp. 86-92).

This gene was mutagenized using the oligonucleotide-directed in vitro mutagenesis system, version 2.1, which is marketed by Amersham (Braunschweig) and based on a method developed by Eckstein (*Nucl. Acids. Res.* (1986) 14, pp. 9679-9698 and *Nucl. Acids. Res.* (1988) 16, pp. 791-802). The mutagenesis was carried out exactly in accordance with the protocol which is enclosed with this Amersham mutagenesis system. The method is summarized below. Details can be obtained from the protocol of this mutagenesis system.

That part of the pUC19-cloned gene for the *Bacillus circulans* #8 β -CGTase which encoded the region, according to the invention, from amino acid residue 155 to amino acid residue 195 of the CGTase was cloned into the commercially available vector

M13 (New England Biolabs) using commercially available enzymes such as restriction endonucleases and T4 DNA ligase (Boehringer, Mannheim). A 1.6 kb AccI fragment is an example of such a fragment. This fragment was cloned into the AccI-cleaved M13 vector.

Single-stranded, recombinant M13 DNA (initial DNA) was isolated, in accordance with the experimental protocol supplied by Amersham together with the above mentioned mutagenesis system, from those *E. coli* host cells which had taken up the recombinant M13 vector.

For the actual mutagenesis, chemically defined mutagenesis oligonucleotides were synthesized which in each case possessed the desired sequence. Such oligonucleotides can, for example, be obtained commercially from MWG (Ebersberg). The sequence of the mutagenesis oligonucleotide was chosen such that the order of the bases in the mutagenesis oligonucleotide was inversely complementary to that part of the nucleotide sequence

of the initial DNA which flanked, for 15 bases upstream and 15 bases downstream, the base triplets of the *Bacillus circulans* #8 β -CGTase which were contained in the initial DNA and which were to be deleted.

The sequence of the mutagenesis oligonucleotide which was used is shown in Table 2.

TABLE 2

5'-(SEQ ID NO:6)-3'

Use of the mutagenesis oligonucleotide shown in Table 2 resulted in a β -CGTase gene fraction which encodes an amino acid fragment from which the six amino acid residues (SEQ ID NO:5) have been deleted.

The mutagenesis oligonucleotide was phosphorylated at its 5' end using T4 polynucleotide kinase and ATP (Amersham). The phosphorylated mutagenesis oligonucleotide was bound to the

homologous regions of the initial DNA. For this, 5 μ g of single-stranded initial DNA were incubated, at 70°C for three minutes and then at 37°C for 30 minutes, together with approximately 4 pmol of the phosphorylated mutagenesis oligonucleotide. A DNA strand which was complementary to the initial DNA, with the exception of the nucleotides to be deleted, was then synthesized, with the mutagenesis oligonucleotide which was bound to the initial DNA serving as the starting point for the synthesis and the initial DNA serving as the template for the new synthesis of the mutated DNA strand. The synthesis itself was carried out, after adding the Klenow fragment of DNA polymerase (Amersham), a T4 DNA ligase and a nucleotide mix which contains the nucleotides dATP, dGTP and dTTP and, in place of dCTP, the thionucleotide dCTPS (Amersham), at 16°C for 15 hours.

Remaining molecules of single-stranded initial DNA were removed from this synthesis mixture. For this, the mixture was treated with NaCl and filtered through a nitrocellulose filter (Amersham) which specifically binds single-stranded DNA.

The double-stranded hybrid DNA, which remained in the flow-through, was concentrated and desalted by precipitation with EtOH. The hybrid DNA was then incubated with NciI (Amersham), a restriction endonuclease which recognizes the nucleotide sequence CC(G/C)GG but only cleaves native DNA strands and not those which contain the nucleotide analog dCTPS, at 37°C for 90 minutes in a suitable incubation buffer (Amersham). This treatment only introduced breaks into the non-mutagenized strand (initial DNA).

The initial DNA was then removed during a 30-minute treatment, at 37°C, with exonuclease III (Amersham), which is an enzyme which degrades DNA strands starting at free ends. After the exonuclease III had been inactivated thermally (70°C for 15 minutes), the remaining, single-stranded and mutagenized DNA strand was incubated with DNA polymerase I (Amersham), T4 DNA ligase and the nucleotides dATP, dTTP, dCTP and dGTP at 16°C for 3 hours. This made the mutagenized single-stranded DNA into double-stranded DNA. Following a further EtOH precipitation for

purification purposes, the mutagenized DNA was transformed into competent *E. coli* K12 cells.

The success of the mutagenesis procedure was checked by sequence analysis of the relevant region of the recombinant DNA from five clones which were obtained in the transformation. The DNA fragment which was originally cloned into M13 for the mutagenesis was excised from a vector, which was confirmed to possess a mutation, using the restriction enzymes XhoI and NdeI.

Subsequently, the corresponding, but non-mutagenized, XhoI/NdeI fragment was excised from the pUC19-based plasmid for expressing *Bacillus circulans* #8 β -CGTase and replaced with the mutagenized fragment using T4 DNA ligase.

EXAMPLE 2Mutagenesis of the *Bacillus sp. 1-1* β -CGTase

The six codons of the *Bacillus sp. 1-1* β -CGTase gene encoding the amino acid residues (SEQ ID NO:7) at position 167-172 of the corresponding CGTase (see Table 1) were deleted, in analogy with the method described in Example 1, using the oligonucleotide shown in Table 3 (A) in Ex. 8, Comparison 2). The same deletion was also introduced into a derivative of this CGTase which was described in DE 43 24 650 A1 and whose γ -CD specificity had already been increased, as compared with that of the wild-type enzyme, by an amino acid exchange (Tyr => Trp) (B) in Ex. 8, Comparison 2).

TABLE 35'-(SEQ ID NO:8)-3'

EXAMPLE 3

Production of *Bacillus circulans* #8 β -CGTase
and its derivatives according to the invention, in *E. coli*

The pUC19-based expression plasmids which were described in Example 1 were transformed into a secretory *E. coli* strain for the purpose of producing the *Bacillus circulans* #8 β -CGTase and its derivative which was prepared as described in Ex. 1. *E. coli* WCM105 was used as the secretory *E. coli* strain. This strain was prepared from *E. coli* DS 410 as described in EP 338,410.

For the purpose of producing the *Bacillus circulans* #8 β -CGTase, or its derivative, therefore, *E. coli* WCM105 cells harboring suitable CGTase expression plasmids were incubated in LB medium (Maniatis, *Molecular Cloning, A Laboratory Manual*; Cold Spring Harbor Laboratory (1982), N.Y.), containing 10 g/l lactose and 0.1 g/l ampicillin at 30°C for 72 hours in a shaking water bath (revolution rate: 250 rpm). The cells were then separated

off by centrifuging at 5000 x g. The cell-free culture supernatant contains the β -CGTase or its derivatives.

EXAMPLE 4

Production of the *Bacillus sp. 1-1* β -CGTase, and its derivatives according to the invention, in *E. coli*

Production was effected utilizing a procedure analogous to that described in Example 3 using the expression plasmids described in Example 2.

EXAMPLE 5

Purification of CGTases by means of adsorption to carrier-bound β -cyclodextrin

CGTases are purified in a specific and mild manner by means of affinity purification using SEPHAROSE®-coupled β -CD molecules.

1 g of epoxy-activated SEPHAROSE® 6B (Sigma) is washed with 3 x 10 ml of H₂O and then with 1 x 5 ml of 0.1 N NaOH. The SEPHAROSE® 6B is subsequently suspended in 2 ml of a 2.8% (w/v) solution of β -CD in 0.1 N NaOH, and this suspension is incubated at 45°C for 20 h while being shaken gently. The coupling product, consisting of β -CD and SEPHADEX® 6B, is then washed with 2 x 5 ml of H₂O. After having suspended the washed material in a 2.5% (w/v) solution of glucose in H₂O, the suspension is incubated at RT for 1 h in order to saturate the free coupling sites which remain. The coupling product is then washed successively with 2 x 5 ml of H₂O, 2 x 5 ml of 0.1 M borate buffer, pH 8.0, 2 x 5 ml of 0.1 M acetate buffer, pH 4.0, and 2 x 2 ml of 20 mM triethanolamine/Cl, pH 7.2. The coupling product is treated with 0.2 ml of 20 mM triethanolamine/Cl, pH 7.2 (final volume, approximately 2-2.5 ml) and stored until used at 4°C.

In order to specifically bind CGTases to the SEPHAROSE® 6B-coupled β -CD (CD-SEPHAROSE®), the cell-free,

CGTase-containing culture supernatants which were obtained in accordance with Example 3 or 4 are treated with 0.2 ml of the CD-Sepharose and incubated at 4°C for 1.5 h while being shaken gently. During this period, the enzyme couples to the CD-SEPHAROSE®. The enzyme/CD-SEPHAROSE® complex is isolated by centrifugation (5 min at 4000 x g) and washed with 2 x 10 ml of 20 mM triethanolamine/Cl, pH 7.2. The CGTase enzyme is subsequently eluted by incubating the complex, at 4°C for 1.5 h, with 2 ml of a 1% solution of β -CD in 20 mM triethanolamine/Cl, pH 7.2. After a final centrifugation (5 min at 4000 x g), the supernatant containing the purified CGTase is removed.

Before characterizing the CGTases which have been purified in this way, the β -CD which is contained in the solution, and which was used for the elution, still has to be removed. To do this, a commercially obtainable PD-10 column (SEPHADEX® G-25 M; Pharmacia) is equilibrated with 35 ml of 20 mM Tris/HCl, pH 7.2 and 5 mM CaCl₂ (TC buffer). The

β -CD-containing solution is made up to a volume of 2.5 ml with TC buffer and loaded onto the column. The column is subsequently eluted with 3.5 ml of TC buffer. The eluate which is obtained in this way contains the purified, β -CD-free CGTase.

EXAMPLE 6

Conversion of starch into cyclodextrins

The CGTase activities were determined using the method described in *Eur. J. Biochem.* (1990) 191, pp. 177-185.

Different quantities of a CGTase solution to be tested were incubated at 45°C, for a defined time, with a 5% solution of a soluble starch (Merck, Darmstadt) in a buffer consisting of 20 mM Tris/HCl, pH 7.2, and 5 mM CaCl₂. After the defined time, the reaction was terminated by adding 1.5 parts by volume of methanol. Unreacted residual starch was precipitated by incubating at 4°C for one hour and separated off by centrifugation (10 min at 12000 x g). The resulting products were

determined by HPLC on a NUCLEOSIL® 10-NH₂ column (Macherey & Nagel, Düren), with defined cyclodextrins (Sigma, Munich) serving as standards. One unit (1 U) is defined as the quantity of enzyme which forms 1 μ M of cyclodextrins per minute from starch under the conditions described.

EXAMPLE 7

Determination of the specific total CGTase activities of purified CGTases

The specific total CGTase activity of purified CGTases is defined as the volume activity per quantity of protein (U/mg).

The CGTase volume activity (U/ml) of an enzyme sample is determined as described in Example 6.

The protein content (mg/ml) of a solution of the purified CGTase is determined using the method described by M.

Bradford (*Anal. Biochem.* (1976) 72, pp. 248 ff). The chemicals which are required for this purpose are obtained from Bio-Rad.

The following specific total CGTase activities (spec. activity) were determined for the *Bacillus Circulans* #8 and *Bacillus sp. 1-1* β -CGTases and the deletion mutants which were produced (as described in Example 1 and Example 2) from them:

<u>Enzyme</u>	<u>Rel. activity (%)</u>	<u>Spec. activity (U/mg)</u>
Wild-type CGTase from <i>Bacillus circulans</i> #8	100	106
Deletion mutant derived from it	77	82
Wild-type CGTase from <i>Bacillus sp. 1-1</i>	100	120
Deletion mutant derived from it	65	78
Mutated derivative (TyrTrp) of <i>Bacillus sp.</i> <i>1-1</i> CGTase	100	23
Deletion mutant derived from it	73	17

EXAMPLE 8

Conversion of starch using the non-mutagenized
 β -CGTases from *Bacillus circulans* #8 and *Bacillus* sp. 1-1 and
the derivatives prepared as described in Exs. 3 and 4.

In order to compare the product spectra of the non-mutagenized CGTases with those of the derivatives which were in each case obtained from them by deletion mutagenesis, as described in Examples 1 and 2, identical enzyme activities were employed for converting the starch (Example 6). At defined times, the product composition was investigated as described. The following results were obtained:

COMPARISON 1: BACILLUS CIRCULANS #8

Time (min)	Wild-Type CGTase				Deletion mutant of this			
	α -CD (%)	β -CD (%)	γ -CD (%)	$\gamma/(\alpha+\beta)$	α -CD (%)	β -CD (%)	γ -CD (%)	$\gamma/(\alpha+\beta)$
5	13	70	17	0.2	9	58.0	33	0.49
10	13.5	70.3	16.2	0.19	8.2	60.5	31.3	0.46
15	11.7	66.3	22.0	0.28	9.4	62.0	28.6	0.40

COMPARISON 2: *BACILLUS SP. 1-1*

A)

Time (min)	Wild-Type CGTase				Deletion mutant of this			
	α -CD (%)	β -CD (%)	γ -CD (%)	$\gamma/(\alpha+\beta)$	α -CD (%)	β -CD (%)	γ -CD (%)	$\gamma/(\alpha+\beta)$
5	0	100	0	0	68	32	33	0.47
10	0	88	12	0.14	0	68	32	0.47
15	0	88	12	0.14	0	67.7	32.3	0.48

B)

Time (min)	Wild-Type CGTase				Deletion mutant of this			
	α -CD (%)	β -CD (%)	γ -CD (%)	$\gamma/(\alpha+\beta)$	α -CD (%)	β -CD (%)	γ -CD (%)	$\gamma/(\alpha+\beta)$
5	0	27	73	2.7	0	0	100	-
10	0	31	69	2.23	0	22	78	3.54
15	0	32.5	67.5	2.08	0	16.6	83.4	5.02

While several embodiments of the present invention have been shown and described, it is to be understood that many changes and modifications may be made thereunto without departing from the spirit and scope of the invention as defined in the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT:
Consortium für elektrochemische Industrie
GmbH whose full post office address is
Zielstattstrasse 20, 81379 München, Germany
- (ii) TITLE OF INVENTION:
Cyclodextrin Glycosyl Transferases
for Producing γ -Cyclodextrin
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
225 Metcalfe Street
Suite 606
Ottawa, Ontario
K1P 1P9
Canada
- (v) COMPUTER READABLE FORM:
 - a) COMPUTER: IBM PC compatible
 - b) OPERATING SYSTEM: PC-DOS/MS-DOS
 - c) SOFTWARE: WordPerfect Version 5.1 for DOS
- (vi) CURRENT APPLICATION DATA:
 - a) APPLICATION NUMBER: Not Yet Known
 - b) FILING DATE: March 13, 1997
 - c) CLASSIFICATION: Not Yet Known
- (vii) PRIOR APPLICATION DATA:
 - a) APPLICATION NUMBER: DE 19615336
 - b) FILING DATE: 18-APR-1996
 - c) CLASSIFICATION: Unknown
- (viii) ATTORNEY/AGENT INFORMATION:
 - a) NAME: McFadden, Fincham
 - b) REFERENCE NUMBER: 1546-281

(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
 - a) LENGTH: 24 Amino acids
 - b) TYPE: Amino acid
 - c) STRANDEDNESS:
 - d) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: No
- (v) FRAGMENT TYPE: Internal fragment

(vi) ORIGINAL SOURCE:
 a) ORGANISM: *Bacillus ohbensis*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
 Pro Asn His Ser Ser Pro Ala Leu Glu Thr Asp Pro Ser Tyr Ala Glu
 1 5 10 15
 Asn Gly Ala Val Tyr Asn Asp Gly
 20

(2) INFORMATION FOR SEQ ID NO: 2:
 (i) SEQUENCE CHARACTERISTICS:
 a) LENGTH: 24 Amino acids
 b) TYPE: Amino acid
 c) STRANDEDNESS:
 d) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iii) HYPOTHETICAL: No

(v) FRAGMENT TYPE: Internal fragment

(vi) ORIGINAL SOURCE:
 a) ORGANISM: *Bacillus macerans*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
 Pro Asn His Thr Ser Pro Ala Asp Arg Asp Asn Pro Gly Phe Ala Glu
 1 5 10 15
 Asn Gly Gly Met Tyr Asp Asn Gly
 20

(2) INFORMATION FOR SEQ ID NO: 3:
 (i) SEQUENCE CHARACTERISTICS:
 a) LENGTH: 24 Amino acids
 b) TYPE: Amino acid
 c) STRANDEDNESS:
 d) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iii) HYPOTHETICAL: No

(v) FRAGMENT TYPE: Internal fragment

(vi) ORIGINAL SOURCE:
 a) ORGANISM: *Bacillus* sp. 1-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
 Pro Asn His Ser Ser Pro Ala Leu Glu Thr Asn Pro Asn Tyr Val Glu
 1 5 10 15
 Asn Gly Ala Ile Tyr Asp Asn Gly
 20

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(viii) POSITION IN THE GENOME:
c) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
CACACCTCTC CAGCGTTTGC CGAAAATGGC 30

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
a) LENGTH: 6 Amino acids
b) TYPE: Amino acid
c) STRANDEDNESS: Single
d) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: Internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Leu Glu Thr Asn Pro Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
a) LENGTH: 30 base pairs
b) TYPE: Nucleotide
c) STRANDEDNESS: Single
d) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
a) DESCRIPTION: /desc = "synthetic"

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(viii) POSITION IN THE GENOME:
c) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
CATTTCATCAC CGGCATATGT TGAAAATGGG 30

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
a) LENGTH: 6 Amino acids

- b) TYPE: Amino acid
- c) STRANDEDNESS: Single
- d) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: Internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Asp Arg Asp Asn Pro Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - a) LENGTH: 6 Amino acids
 - b) TYPE: Amino acid
 - c) STRANDEDNESS: Single
 - d) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: Internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Leu Glu Thr Asp Pro Ser
1 5

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A CGTase protein which converts starch substrates to CD, produces γ -CD to an increased extent relative to the wild-type and which still exhibits at least 60% of specific total γ -CGTase activity of the starting CGTase which was used for preparing it, comprising

CGTase having an amino acid sequence differing from the amino acid sequence of starting CGTase by deletion of at least one amino acid in a region from amino acid position 155 up to and including amino acid position 195,

where position 1 of protein sequence is the beginning of a signal peptide for the CGTase and deletion brings about an increase in the γ -CGTase activity of the protein.

2. The CGTase protein as claimed in claim 1,

wherein between four and eight amino acids have been deleted from the region between amino acid position 155 and amino acid position 195 of the protein sequence,

where position 1 of the protein sequence is the beginning of the signal peptide of the CGTase and the deletion brings about an increase in the γ -CGTase activity of the protein.

3. The CGTase protein as claimed in claim 1,

wherein six amino acids have been deleted from the region between amino acid position 155 and amino acid position 195 of the protein sequence,

where position 1 of the protein sequence is the beginning of the signal peptide of the CGTase and the deletion brings about an increase in the γ -CGTase activity of the protein.

4. The CGTase protein as claimed in claim 1,

wherein the amino acid sequence differs from the amino acid sequences of the CGTases specified in FIG. 1 at least by the deletion of the amino acid residues which are in each case printed in bold in FIG. 1, and is selected from the group consisting of

(SEQ ID NO:5) deleted from (SEQ ID NO:4);
(SEQ ID NO:7) deleted from (SEQ ID NO:3);
(SEQ ID NO:9) deleted from (SEQ ID NO:2);
(SEQ ID NO:10) deleted from (SEQ ID NO:1);

where the remaining amino acid sequence of the CGTase is homologous to the amino acid sequence of the CGTase from the microorganism which is in each case specified in FIG. 1 to the extent that the sequence exhibits CGTase activity without the deletion according to the invention.

5. A process for preparing a mutated CGTase gene which encodes a CGTase comprising

mutating a DNA sequence of a starting gene encoding a starting CGTase to produce a mutated gene,

such that an amino acid sequence encoded by a DNA sequence of the mutated gene differs from an amino acid sequence encoded by the DNA of the starting unmutated gene by the deletion of at least one amino acid from a region between amino acid position 155 and amino acid position 195.

6.

6. The process as claimed in claim 5, comprising

mutating a DNA sequence of a starting gene encoding a starting CGTase to produce a mutated gene,

such that an amino acid sequence encoded by a DNA sequence of the mutated gene differs from an amino acid sequence encoded by the DNA of the starting unmutated gene by the deletion of from four to eight amino acids from a region between amino acid position 155 and amino acid position 195.

7. The process as claimed in claim 5, comprising

mutating a DNA sequence of a starting gene encoding a starting CGTase to produce a mutated gene,

such that the amino acid sequence encoded by the DNA sequence of the mutated gene differs from an amino acid sequence encoded by the DNA of the starting unmutated gene by the deletion of six amino acids from a region between amino acid position 155 and amino acid position 195.

8. A DNA sequence, which encodes a CGTase as claimed in claim 1.

9. A process for preparing a γ -CGTase, comprising

expressing at least one DNA sequence as claimed in claim 8 in a microorganism.

10. In a process for preparing γ -CD by converting starch using a CGTase,

the improvement which comprises utilizing at least one CGTase as claimed in claim 1 as the CGTase.

11. In a process for preparing γ -CD by converting starch using a CGTase,

the improvement which comprises utilizing at least one CGTase as claimed in claim 2 as the CGTase.

12. In a process for preparing γ -CD by converting starch using a CGTase,

the improvement which comprises utilizing at least one CGTase as claimed in claim 3 as the CGTase.

13. In a process for preparing γ -CD by converting starch using a CGTase,

the improvement which comprises utilizing at least one CGTase as claimed in claim 4 as the CGTase.

FIG. 1

<u>β-CGTase from</u>	<u>Position</u>	<u>Amino acid sequence</u>	
<i>Bacillus ohbensis</i>	160	-PNHSSPA LETDPS	YAENGAVYNDG- (SEQ ID NO:1)
<i>Bacillus macerans</i>	165	-PNHTSPA DRDNPG	FAENGGMVDNG- (SEQ ID NO:2)
<i>Bacillus sp. 1-1</i>	160	-PNHSSPA LETNPN	YVENGAIYDNG- (SEQ ID NO:3)
<i>Bacillus circulans</i> #8	172	-PNHTSPA METDTS	FAENGRLYDNG- (SEQ ID NO:4)